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The sunflower transcription factor HaHB11 improves yield, biomass and tolerance to flooding in transgenic Arabidopsis plants

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ABSTRACT

HaHB11 is a member of the sunflower homeodomain-leucine zipper I subfamily of transcription factors. The analysis of a sunflower microarray hybridized with RNA from *HaHB11*-transformed leaf-disks indicated the regulation of many genes encoding enzymes from glycolisis and fermentative pathways. A 1300 bp promoter sequence, fused to the *GUS* reporter gene, was used to transform Arabidopsis plants showing an induction of expression after flooding treatments, concurrently with *HaHB11* regulation by submergence in sunflower. Arabidopsis transgenic plants expressing *HaHB11* under the control of the CaMV 35S promoter and its own promoter were obtained and these plants exhibited significant increases in rosette and stem biomass. All the lines produced more seeds than controls and particularly, those of high expression level doubled seeds yield. Transgenic plants also showed tolerance to flooding stress, both to submergence and waterlogging. Carbohydrates contents were higher in the transgenics compared to wild type and decreased less after submergence treatments. Finally, transcript levels of selected genes involved in glycolisis and fermentative pathways as well as the corresponding enzymatic activities were assessed both, in sunflower and transgenic Arabidopsis plants, before and after submergence. Altogether, the present work leads us to propose HaHB11 as a biotechnological tool to improve crops yield, biomass and flooding tolerance.

1. Introduction

Food production is affected by abiotic factors worldwide. Among abiotic stressing conditions, flooding is one of those causing the most severe constraints (Boyer, 1982). Soil can be flooded due to a poor drainage or to an excessive rainfall or irrigation. The severity of the damage caused on plants depends upon stress duration in time, crop variety, developmental stage, soil type, fertility levels, presence of pathogens and levels of water excess (Sullivan et al., 2001). Flooding stress initiates by either waterlogging, when only the roots are flooded, or complete submergence when the entire plant is under water. To overcome the lethal effects of submergence, plants have evolved two different strategies: the low-oxygen escape and the low-oxygen quiescence. In the escape strategy, plants elongate their internodes in order to reach the surface and restore air contact (Bailey-Serres and Voesenek, 2008). Plants adopting this strategy often have more aerenchyma tissue for efficient gas transport, especially to the below-ground parts where the hypoxia effects are most severe (Sauter, 2000; Voesenek et al., 2003). In the quiescence strategy, plants reduce their growth to conserve their reserves, thus causing a delay in the energetic crisis (Bailey-Serres and Voesenek, 2008; Sauter, 2000; Setter and Laureles, 1996). In rice, the ethylene response factor (ERF) genes, *SNORKEL* and *SUB1,* have been shown to play a crucial role in submergence tolerance and respectively control the escape and quiescent strategies in different varieties (Fukao et al., 2006; Gibbs et al., 2011; Hattori et al., 2007, 2008, 2009; Xu et al., 2006). On the other hand, in Arabidopsis *HRE1* and *HRE2* (Hypoxia Responsive ERF genes), have been described as playing a role in low oxygen signaling, improving flooding tolerance by enhancing anaerobic gene expression and ethanolic fermentation (Licausi et al., 2010).

Besides the above mentioned tolerance strategies, the initial cellular response of plants, when are flooded, is to promote the anaerobic metabolism of pyruvate. Adaptation to oxygen deficiency (anoxia or hypoxia) in flooded soils engages a combination of morphological and metabolic processes, involving several enzymatic systems (Fox et al., 1995). Oxygen deficiency inhibits aerobic respiration and starch mobilization, concurrently inducing anaerobic fermentation pathways (Crawford, 1992). Metabolic responses to

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hypoxia can be considered active or passive in terms of carbohydrate consumption. The carbohydrate consuming response leads to the catabolism of soluble carbohydrates to generate ATP through glycolysis and to regenerate NAD^+ through ethanolic fermentation. This energy is used to promote the growth of submerged organs through ethylene production, which stimulates giberellins-dependent stem internode and petiole elongation. On the other hand, the response conserving carbohydrates retains reserves of starch and hexoses, necessary for aerobic metabolism upon stress release (Fukao and Bailey-Serres, 2004).

The ability to survive to flooding, as well as to any other stress condition, constitutes an important valuable trait. However, the adaptive responses leading to survival frequently cause detrimental consequences, which vary depending on the strength and duration of stress. Among these detriments, the most common are impairment of photosynthesis, and reduction of biomass and yield (and even plant death when the stress is severe or/and prolonged) (Rivero et al., 2007; Skirycz et al., 2011). In improvement programs, penalties should be evaluated because survival rates, via any plant adaptive strategy, are certainly relevant but this likely not correlates with the enhancement of yield or biomass (Skirycz et al., 2011).

Transcription factors (TFs) play crucial roles in plant adaptation to adverse environmental conditions but only a few TFs have been associated to the flooding response. MYC, MYB, AP2/ERF and NAC members have been reported as able to up-regulate alcohol dehydrogenase (ADH) expression in Arabidopsis (Abe et al., 2003; Christianson et al., 2009; Papdi et al., 2008) and MYB2 has been directly related to hypoxic stress (Hoeren et al., 1998). More recently, three NF-YA family members were also related to flooding tolerance (Leyva-González et al., 2012). Among plant TFs, the homeodomain-leucine zipper I (HD-Zip I) subfamily members have been proposed as developmental regulators involved in abiotic stress responses (Henriksson et al., 2005; Schena and Davis, 1994). There are many reports supporting this role assignment, most of them related to the drought response (Ribichich et al., 2014). However, HD-Zip I TFs have not been related to response to stress by flooding so far. Some sunflower TFs have been identified as potential biotechnological tools to improve crops. Among them, there are the examples of the divergent HaHB4 conferring drought and salinity tolerance and the conserved HaHB1, which confer tolerance to freezing temperatures and drought (Cabello et al., 2012; Cabello and Chan, 2012; Dezar et al., 2005). Notably, these TFs from the Asteraceae family did not conduct to yield penalties when they were expressed as transgenes in Arabidopsis and other crops (Cabello et al., 2010; Cabello and Chan, 2012; Chan et al., 2013). HaHB11 is a sunflower TF belonging to the HD-Zip I subfamily. It exhibits similarity with members of clade IV, but the fact that it was not resolved in this clade supports that is a divergent member of the family (Arce et al., 2011). Divergent TFs could explain, at least in part, the different abilities and adaptive responses exhibited by diverse plant species. In this sense, sunflower, belonging to the Asteraceae, is a species able to grow in dissimilar agronomic situations, particularly on soils with different levels of water (Quartacci and Navari-Izzo, 1992). Herein, we report that HaHB11 likely participates in the flooding response in sunflower and that its ectopic expression in Arabidopsis plants significantly improves yield and tolerance to flooding.

2. Material and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana Heyhn Columbia (Col0) and *Helianthus annuus* cv. HA89 were grown in Klasmann Substrat No. 1 compost (Klasmann-Deilmann GmbH, Germany) at 22–24 °C under long-day photoperiods (16 h of illumination; 150 $\mu E\ m^{-2}\ s^{-1}$). *A. thaliana* were grown in Petri dishes containing 0.8% agar—Murashige and Skoog (MS) medium or in soil pots (300 cm^3). Sunflowers seedlings were grown in 300 cm^3 soils pots and at stage V2 placed in 10 l pots.

2.2. RNA isolation and expression analyses by real time RT-PCR

RNA for RT-qPCR was extracted with TRIzol® reagent (Invitrogen™) according to the manufacturer instructions. RT-PCR analyses were conducted essentially as described previously (Cabello et al., 2012). RNA levels were determined by normalization with *ACTIN* and *UBIQUITIN* transcript levels according to the $\Delta\Delta$Ct method. Three biological replicates, tested by triplicate, were used to calculate standard deviation. Differences were considered significant when the p-values were <0.05 (Student's *t*-test). Specific oligonucleotides for each gene were designed and shown in Supplementary file 6 (primerblast).

2.3. Genetic constructs

2.3.1. 35S:HaHB11

The *HaHB11* cDNA was amplified by PCR using as template an EST obtained from Tucson University, Arizona (http://www2.genome.arizona.edu; NCBI Accession No. DY923855) and the oligonucleotides H11F and H11R (Supplementary file 6) bearing respectively *Bam*H1 and *Sac*I sites. The amplification product was cloned into the pBI121 plasmid.

2.3.2. ProHB11:HaHB11

The *HaHB11* promoter, 1363 bp upstream the transcription start site, was isolated from a genomic BAC (Clemson University – Id: 175N08, library HA_HBa from *H. annuus* ecotype HA383), amplified with PromF and PromR bearing *Hind*III and *Xba*I sites respectively, and cloned into the construct *35S:HaHB11* by replacing the CaMV 35S promoter.

2.3.3. ProHB11:intron:HaHB11

An *Xba*I/*Xba*I fragment containing the Arabidopsis *COX*5c-2 leader intron (Cabello et al., 2007) was cloned into the *Xba*I site of the *ProHB11:HaHB11* clone between the promoter and the coding sequence.

2.3.4. ProHB11:GUS

The *HaHB11* promoter fragment isolated as described above was cloned in the *Hind*III/*Xba*I sites of the pBI101 vector.

2.4. Stable transformation of Arabidopsis plants

A transformed *Agrobacterium tumefaciens* strain, LBA4404, was used to transform Arabidopsis plants by the floral dip procedure (Clough and Bent, 1998). Transformed plants were selected on the basis of their resistance. Transgene insertions were verified by PCR. Lines with one copy of the transgene were selected by segregation and three/four independent lines were further reproduced. Homozygous T3 and T4 plants were used to analyze transgene expression levels and plant phenotypes.

2.5. Flooding treatments

Submergence: 25-day-old Arabidopsis or V3 (Schneiter and Miller, 1981) sunflower plants were completely submerged in large trays with 3 cm of water over the aerial part of the plants during 6–8 days in standard illumination conditions (see above). Waterlogging assays: only the roots were submerged by filling the tray in which the 8 cm height pots were placed with water to a height